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Evidence for speciation within nominal *Pontia daplidice* (LINNAEUS, 1758) in southern Europe (Lepidoptera : Pieridae)

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Summary

Further evidence for the existence of two genetically distinct taxa within nominal *Pontia daplidice* L. in southern Europe is presented. The two taxa show no common alleles at one locus and only very infrequently at two other loci. These data strongly suggest an interruption of gene flow. *P. daplidice* is in its entire range known as a migratory species and the G_{ST} values indicate that this is likely to be the case for both new taxa. These values also show a low genetic differentiation among populations within each taxon. Initial artificial hybridization experiments with individuals from geographically distant populations provide no indication of a precopulative barrier, but show a remarkable reduction of the viability of the offspring of the first hybrid generation. Such a situation is not known for a species with an open population structure and observations in zones of possible contact promise to be of high interest.

Résumé

Nous présentons ici de nouveaux arguments en faveur de l'existence de deux entités génétiquement distinctes à l'intérieur de *Pontia daplidice* en Europe méridionale. A un locus (sur 22 étudiés par électrophorèse), les deux taxa ne montrent jamais d'allèle en commun et, à deux autres, ce n'est que rarement qu'il en est observé. Ces données représentent une forte indication en faveur d'une interruption du contact génétique. *P. daplidice* est, dans l'ensemble de son aire, une espèce intensément migratrice et les valeurs de G_{ST} indiquent qu'il en est ainsi pour les deux nouveaux taxa. A l'intérieur des deux entités détectées, la différenciation génétique est faible. Par ailleurs, des expériences de croisement entre des populations éloignées des deux taxa montrent à la fois une absence de barrières précopulatoires et une inviabilité considérable dès la première génération hybride. Une telle situation est à l'heure actuelle inédite chez des espèces à structure ouverte. Des études dans les zones de contact potentielles s'annoncent très intéressantes.

Zusammenfassung

In der vorliegenden Publikation werden weitere Befunde präsentiert welche die Existenz zweier genetisch unterscheidbarer Taxa innerhalb *Pontia daplidice* L. in Südeuropa belegen. Die beiden Taxa haben an einem Locus keine, an zwei weiteren Loci nur sehr selten gemeinsame Allele. Diese Daten deuten auf einen unterbrochenen Genfluss hin. *P. daplidice* ist, in der Gesamtheit des Verbreitungsgebietes, als Wanderfalter bekannt. Die G_{ST} Werte deuten darauf hin, dass dies für beide neuen Taxa zutrifft und dass die genetische Differenzierung innerhalb beider Taxa gering ist. Erste Kreuzungsexperimente zwischen entfernten Populationen zeigen einerseits keine präkopulativen Schranken auf, andererseits aber eine starke Reduktion der Ueberlebensraten der Nachkommen der ersten Hybridgeneration. Eine solche Situation ist für eine Art mit offener Populationsstruktur kaum bekannt. Untersuchungen in potentiellen Kontaktzonen sind deshalb sehr vielsprechend.

Introduction

The observation that there are two genetically distinct taxa among the well known southern European *Pontia daplidice* (Linnaeus, 1758) populations (Geiger and Scholl, 1982) came as a surprise to most lepidopterists. This discovery was based on enzyme electrophoretic data that demonstrated the existence of two groups of populations having no common alleles at two loci out of 22 analyzed. There was hardly another interpretation than to conclude that the two taxa are in nature reproductively isolated and differentiated at the species level. We showed that species 1 (*daplidice* L., sensu Wagener, 1988), the western European species, flies on the Canary Islands, Morocco, France, and Israel. Species 2 (*edusa* Fabricius 1777, sensu Wagener, 1988), the eastern European species, in Switzerland, Italy, Yugoslavia, and Greece (Geiger and Scholl, 1982).

These observations (l.c.) raised at least two problems : i) It was not possible to report data from localities where both species occur in sympatry. A direct demonstration of the interruption of gene-flow under this situation was therefore not possible. Furthermore, there were no data from artifical hybridization experiments that would provide at least indirectly, additional arguments. ii) It was not clear whether it is possible to distinguish the two species by means of morphological characters.

The advantages and disadvantages of the use of enzyme electrophoretic data to discuss problems in systematics of European butterflies have been misunderstood several times (e.g. Eitschberger, 1983). The method is a genetical one. It is based on the fact that the genetic information of all organisms is stored in the genetical material, the DNA molecule. The DNA molecule is organized into a number of units, the loci. Starting from the DNA the information is transcribed and translated in a very direct way into

the amino acid sequence of polypeptides. Each locus is responsible for an individual type of polypeptide. A change in the sequence of the nucleotides of the DNA is very often reflected by a change in the amino acid sequence of the polypeptide determined at that locus (the presence of a different allele). These changes may also alter the physico-chemical properties of the polypeptide. Therefore, investigations on the structure of the polypeptides enables the collection of data on the genetic composition of an individual organism. This work is important because the chance that there are such genetic differences between specimens of different taxa is correlated to a high degree with the systematic rank of the taxa (BERLOCHER, 1984 ; for Pieridae see GEIGER, in press). The more distantly related two taxa are, the more often one finds alternative alleles. These different alleles do not directly influence speciation, but they accumulate by chance *as a result* of the interrupted gene-flow after the speciation process.

Working with population samples of several taxa one obtains two kinds of information : i) qualitative information (which alleles can be found at one or several loci), and ii) quantitative information (in what frequencies are the alleles observed).

i) Qualitative information : This kind of information is especially important for argumentation at the species level. If populations of two taxa show different alleles at one or several loci in areas of allopatry but share a common polymorphism in areas of sympatry, this fact is a strong argument for reproductive contact in sympatry and the existence of one species (for butterflies see e.g. GEIGER and REZBANYAI, 1982). On the other hand, if the two taxa show no common polymorphism in this situation in sympatry, this is a strong argument for reproductive isolation and the existence of two species.

ii) Quantitative information : Allopatric taxa are always very problematic to be ranked. In such situations only the degree of genetic differentiation may provide us with reliable data for argumentation. Enzyme electrophoretic data can be quantified e.g. by calculating I-values (NEI, 1972) that are a measure for the degree of genetic similarity between two taxa. I-values range between 0 and 1. An I-value of 0.80 can be regarded in a rough approximation as an indication that the two taxa show 80% correspondance in their alleles and the allelic frequencies. For a given group of organisms there is in general a good correlation between the I-value and the taxonomic rank (BERLOCHER, 1984. For Pieridae see GEIGER, in press).

In principle, enzyme electrophoretic data are not different from classically collected morphological data. The advantage is that the genetic variants are easily distinguishable. Such an analytical power can hardly be reached with "classical" methods. Electrophoretic variants are, for most practical purpo-

ses, not detectably modified epistatically. Modifications by environmental factors, a major problem in "classical" butterfly morphology, can practically be excluded. Of course, as any other method, enzyme electrophoresis also has its disadvantages. It is, e.g., possible that in rapidly evolving groups time was too short to allow accumulation of differences in the alleles at the loci covered with this method. However, if there are such differences in or near zones of possible contact, this method provides us with argumentative power hardly reached by "classical" methods. For a more profound discussion of enzyme electrophoretic methods see BERLOCHER (1984) and, especially for butterflies, GEIGER (in press).

It is the purpose of this publication to update the enzyme electrophoretic data for the *daplidice* case, to show that there is evidence for hybrid sterility in laboratory crosses, and to document that there are morphological characters that allow, although not unambiguously, identification of the two species in a number of cases.

Material and Methods

Table 1 gives the origin of the samples used for enzyme electrophoresis and the number of specimens in each sample. The wings and genitalia of all individuals were saved for postelectrophoretic morphological analysis. The specimens were stored at -30°C or -80°C until electrophoresis. Some specimens arrived dead in Bern, but still proved to be suitable for electrophoresis (for controls see GEIGER, 1981 ; GEIGER and SCHOLL, 1982).

Table 1 : Origin of the individuals used for electrophoresis

Pontia daplidice L. : *Spain* : Canary Islands : Gran Canaria, July 1979 (7 individuals), Tenerife, 7.1983 (11), Madrid : Campo Real, 6.1983 (17), Castellon : La Barona, 10.1985 (6). *Morocco* : Marrakech : Taddert, 10.1981 (3). *France* : Pyrénées orientales : Banyuls-sur-mer, 10.1981 (11) and 9.1982 (14), Le Barcares, 9.1982 (3), Herault : Agde, 10.1981 (4), Colombier, 6.1979 (1), Gard : Nimes, 9.1984 (10), Le Grau-du-Roi, 9.1983 (19), Ardèche : Orgnac l'Aven, 6.1979 (5), Alpes Maritimes, Col de Vence, 7.1981 (3), Corse : Bastia, 5.1983 (5), San Pellegrino, 5.1983 (4). *Turkey* : Hakkari : Zab-Tal, 25 km NNW Cukurca, 6.1985 (1). *Israel* : Revivim, 10.1980 (3), The Negev Central Mtn., 5.1982 (2).

Pontia edusa FABRICIUS 1777 : *Switzerland* : Wallis : Brig and surroundings, 4. and 5.1979 (3), 6.1981 (1), 8.1983 (3), 5.1984 (1), Sion, 7.1981 (1), Saas Almagell, 4.1982 (1). *Italy* : Toscana : Rossignano, 7.1981 (1), Emilia-Romana : Modena, 5.1981 (1), Sicily : Randazzo, 4.1982 (2), Puglia : Foggia, 9.1978 (1), Lazio : Rome, 9.1981 (13). *Yugoslavia* : Croatia : Rovinj, 5.1982 (14), Biograd, 5.1982 (6), Serbia : Pristina, 9.1981 (3). *Austria* : Vienna, 7.1982 (5). *Hungary* : Hortobagy : Hortobagy, 4.1983 (13). *Greece* : Thessaloniki, 8.1980 (7). *Turkey* : Hakkari : Suvarihalil (1), Gaziantep (5), Canakkale : Ecebat (1), Denizli : Pamukkale (2), Elazig : Hazar Gölü (1), Urfa : Sirerek (1), Birecik (1), Bitlis : Tatvan (1), all 6.1985.

Enzyme electrophoresis followed standard procedures described elsewhere (GEIGER, 1981 ; GEIGER and SCHOLL, 1985). Twenty-two loci were scored : adenylate kinase (AK-1, AK-2), aldolase (ALD), arginine kinase (APK), fumarase (FUM), glutamate-oxaloacetate transaminase (GOT-1, GOT-2), glutamate-pyruvate transaminase (GPT), glyceraldehyde-phosphate dehydrogenase (GAPDH), α-glycerophosphate dehydrogenase (α-GPDH), hexokinase (HK), indophenol oxydase (IPO), isocitrate dehydrogenase (IDH-1, IDH-2), malate dehydrogenase (MDH-1, MDH-2), malic-enzyme (ME-1, ME-2), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6-PGD), phosphoglucose isomerase (PGI), and pyruvate kinase (PK).

The genetic interpretation of the zymograms is based on the analysis of the progeny of parents with various electrophoretic phenotypes in *Pieris brassicae* L. (GEIGER, 1982). The observed electrophoretic pattern in all individuals investigated in his study showed no deviation from the pattern observed in *P. brassicae*. The distribution of the alleles is also in good accordance with Hardy-Weinberg expectations (in samples with $n \geqslant 10$).

The designation of the alleles is based on *Pieris brassicae* as a standard. The most frequent (common) allele in this species has the index 100. Electromorphs (alleles) with different electrophoretic mobilities are designated in relation to this standard. An enzyme variant migrating 5 mm faster than the common *P. brassicae* variant thus being labeled 105.

The statistic I (NEI, 1972) was used to estimate the genetic similarity between the samples ($n \geqslant 7$) over all loci. The value varies between 0 and 1. An I-value of 1.0 is calculated if all individuals of two compared populations show at all loci the same alleles in the same frequencies ; an I-value of 0 is calculated if the two samples share no common alleles. A dendrogram was constructed by cluster analysis (UPGMA method, see FERGUSON, 1980). Such a dendrogram shows in a graphic way the degree of genetic similarity between taxa found at the loci investigated. To measure the amount of genetic variation among local populations the coefficient G_{ST} (NEI, 1975) was used. A G_{ST} value of 0 is calculated for a set of populations if all the observed genetic variation is due to intrapopulation variation. (For the calculation of G_{ST} see footnote (*). G_{ST} is the mean over all polymorphic loci (GOT-1, GPT, IDH-2, MDH-1, 6-PGD, PGI, PGM).

The specimens used for the intertaxa hybridization experiments were collected in the following localities : *edusa* : Delphi, Greece, April 1986. *da-*

(*) G_{ST} is calculated as $(H_T - \bar{H}_S)/H_T$, where H_T is the total heterozygosity among local populations ($H_T = 1 - \sum \bar{p}_i^2$, where $\bar{p}_i^2$ is the square of the average frequency of the ith allele), and $\bar{H}_S$ is the total heterozygosity within a local population ($\bar{H}_S = 1 - \sum p_i^2$).

The specimens used for the intertaxa hybridization experiments were collected in the following localities : *edusa* : Delphi, Greece, April 1986. *daplidice* from 3 females from the Collines de l'Étoile, near Marseille, France, May 1986. As the *edusa* were out of phase with the *daplidice* it was necessary to breed an F_1 for the first taxon. The butterflies were allowed to fly in a cage (60 × 60 × 60 cm) for the hybridization experiments. Four different experiments were started : i) 1 male *daplidice* (wild, 25 km from Marseille) with 4 females *edusa*. ii) 10 males *daplidice* with 10 females *edusa*; iii) 20 males *daplidice* with 20 females *edusa*. iv) 5 males *edusa* with 5 females *daplidice*.

The wings and genitalia of the electrophoretically identified specimens were retained for morphological investigations. The wing pattern and the male and female genitalia were compared.

Results

At most loci the two taxa show the same alleles in similar frequencies. No genetic variation has been found at eight loci (AK-1, AK-2, ALD, APK, FUM, GADPH, IPO, PK). Variation is very low (f common allele ⩾ .95) at seven other loci (GOT-1, GOT-2, α-GPDH, HK, IDH-1, MDH-2, ME-1). There is considerable polymorphism at four loci (IDH-2, MDH-2, PGI, PGM). The frequencies of the alleles at these loci are not significantly different from those reported earlier on smaller sample sizes (Geiger and Scholl, 1982). Table 2 documents the alleles and their frequencies found at the three diagnostic loci (GOT-1, 6-PGD, and GPT). At the GOT-1-locus the allele 85 is fixed in *edusa*. There are two alleles found in the *daplidice* samples, the allele 93 being the common allele of this taxon. Only two individuals were heterozygous at this locus in *daplidice*, none showed the allele 85 observed in *edusa*. At the GPT-locus the allele 130 is found with a frequency of 0.98 and the allele 125 with a frequency of 0.02 in *daplidice*. The common allele of *daplidice* (allele 135) did not occur in all samples of *edusa* with the exception of one heterozygous individual from Thessaloniki (Greece). The situation is similar at the 6-PGD-locus where the common allele 95 of *daplidice* was found only once in a heterozygous individual from Hortobagy (Hungary).

For both taxa the degree of genetic variation within the taxa is low. Heterozygosity in *daplidice* is 0.059 and in *edusa* 0.053 at the loci investigated. Most of this variation is due to intrapopulation variation as is shown by the low G_{ST}-values among the samples with at least ten individuals (G_{ST} for *daplidice* = 0.047 (five samples : Tenerife, Campo Real, Banyuls-sur-mer, Nimes, Le Grau du Roi) and for *edusa* = 0.044 (three samples : Rome, Rovinj, Hortobagy). As a consequence the degree of genetic similarity within the taxa is very high (I ⩾ 0.97) and all samples of each taxon cluster at these

Table 2
Allelic frequencies at diagnostic loci (samples with n ⩾ 5)

		GOT-1			6-PGD					GPT		
	n	85	93	102	87	95	100	103	108	125	130	135
Gran Canaria	7		1.0			1.0					1.0	
Tenerife	11		1.0			1.0					1.0	
Madrid	17		1.0			1.0				.03	.97	
La Barona	6		1.0			1.0					1.0	
Banyuls-sur-mer	25		.96	.04		.98		.02			1.0	
Nimes	10		1.0			1.0					1.0	
Le Grau du Roi	19		1.0			1.0				.05	.95	
Orgnac l'Aven	5		1.0			1.0				.20	.80	
Bastia	5		1.0			1.0					1.0	
daplidice total	129		.99	.01		.98		.02		.02	.98	
Rome	13	1.0					1.0					1.0
Rovinj	14	1.0					1.0					1.0
Biograd	6	1.0					.67		.33			1.0
Wien	5	1.0					.80		.20			1.0
Hortobagy	13	1.0			.08	.04	.85		.04			1.0
Thessaloniki	7	1.0			.07		.79		.14		.07	.93
Gaziantep	5	1.0					1.0					1.0
edusa total	89	1.0			.02	.01	.93		.05		.01	.99

high levels in the dendrogram (Fig. 1). Due to the much higher degree of genetic differentiation observed between the taxa these two entities cluster at a much lower level of genetic similarity (I = 0.84).

Fig. 2 shows the localities from which the two taxa are known at present. The general picture that was already indicated in the earlier publication (GEIGER and SCHOLL, 1982) is confirmed here. The taxon *daplidice* is known from northern Africa and south-western Europe, the taxon *edusa* from south-eastern Europe. No region of cohabitation has been found until present. However, both taxa occur in places geographically very close in the area between Genoa and Nice (Riviera) and in south-eastern Turkey. there are no individuals found so far that are heterozygous at any of the diagnostic loci for an allele of the other taxon recorded from these areas.

The results of the intertaxa hybridization experiments show that there are no premating barriers between the taxa. In three of the four experiments spontaneous copulations were observed. However, there is evidence for strong postmating barriers. In the first test (1 male *daplidice* with 4 female *edusa*) 7 eggs were laid by the mated female. No larvae hatched and the eggs showed no sign of development. In the second test (10 male *daplidice* with 10 female *edusa*) six pairings were observed within the first half hour. Four females laid some 250 eggs, no larvae hatched, no sign of development of

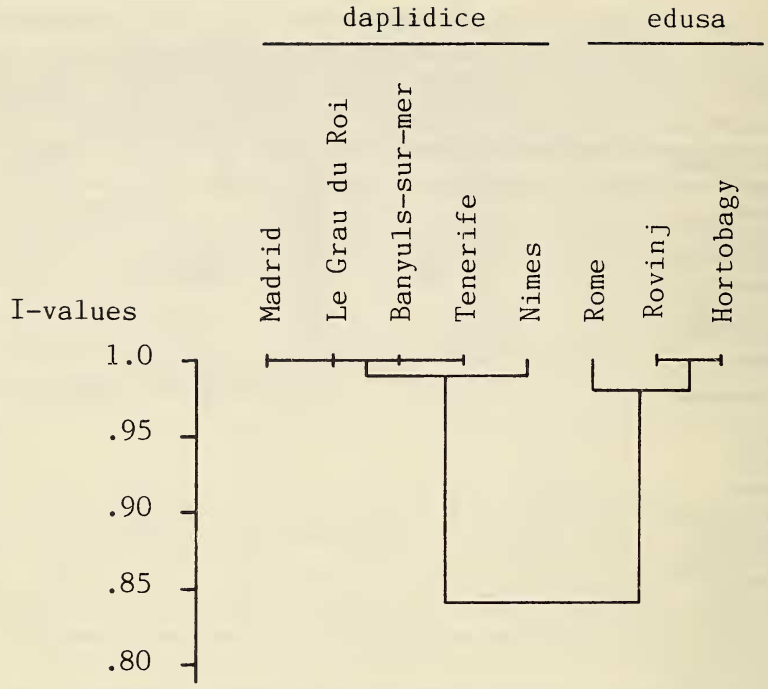
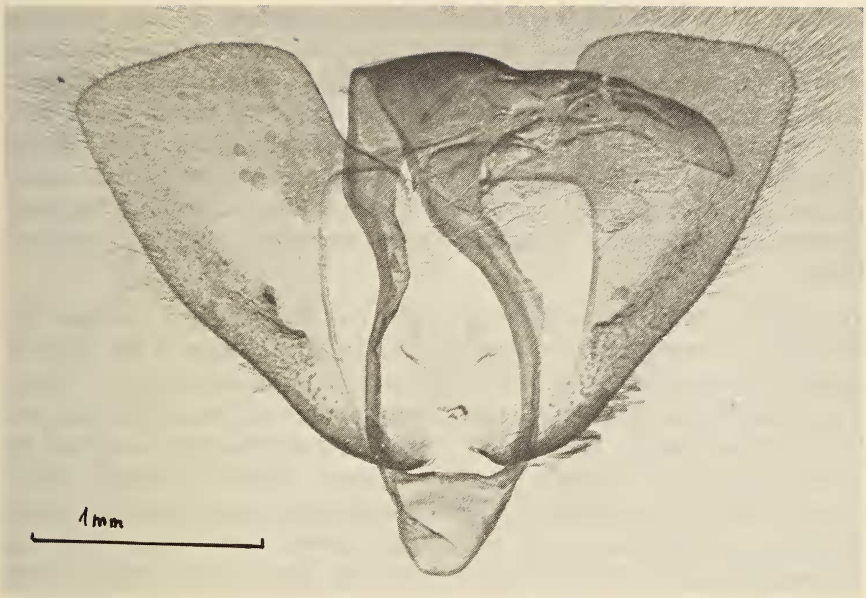
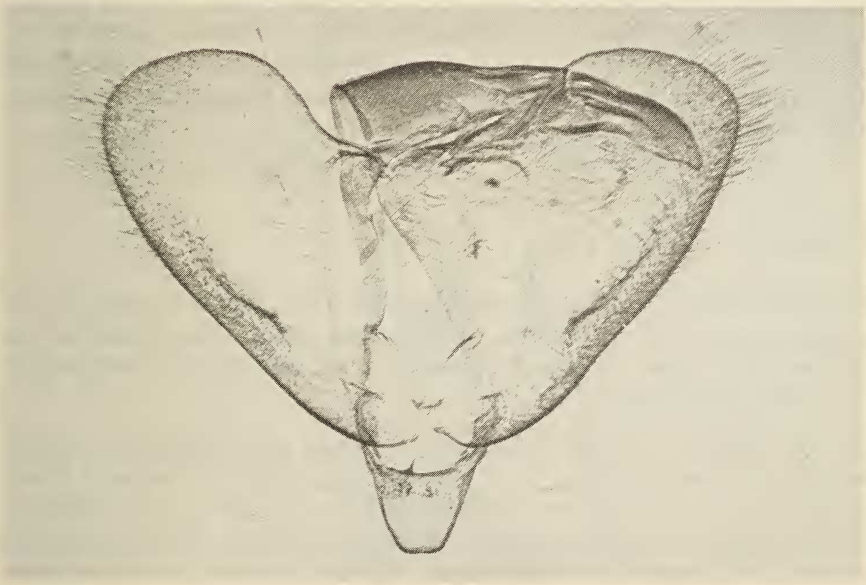


Figure 1. Dendrogram of population samples ($n \geqslant 10$).

Figure 2. Distribution of electrophoretically analyzed population samples of ▲ *P. edusa* and ● *P. daplidice.*



a



b

Figure 3. Male genitalia of *Pontia daplidice* and *P. edusa*. 3a : *Pontia edusa*, Greece, Delphi. 3b : *Pontia daplidice*, France, Marseille (same scale as 3a).

the eggs. In the third test (number of matings unobserved, 20 male *daplidice* with 20 female *edusa*) a high number of eggs were laid, but again no larvae hatched. In the fourth test (5 male *edusa* with 5 female *daplidice*) three pairings were observed during the first hour. Only one female laid a high number of eggs (145) of which 9 larvae hatched and developed to 7 adults (3 males and 4 females). Attempts to have them mated were unsuccessful. Among the 136 unhatched eggs most (some 125) showed signs of incipient development. Dissection of eggs revealed various stages of embryo development.

The analysis of adult morphology of the two taxa revealed evidence for differences in two characters. First, we found that the shape of the valvae is angled at its distal margin in *edusa*, but rounded in *daplidice* (Fig. 3). There is considerable variation in this character in all populations of both taxa and there is some overlap (Fig. 4). This result is independently confirmed by Ackery and Vane-Wright (British Museum (Natural History), pers. comm.) and Wagener (1988). The second distinguishing character in a high number of individuals is the black spot between vein 3 and 4 in the submarginal region of the forewing underside. This spot is either i) well expressed, ii) scarcely visible, or iii) not present. Figure 5 shows that this spot is well developed in some 70% of the *daplidice* males and some 50% of the females in this species, but only in 3% of the males and 5% of the females in *edusa*. Again, there is considerable overlap. Therefore, there is no unequivocal morphological character available at the moment to distinguish the two taxa morphologically. The hybrids showed intermediate conditions both in genitalia and wing pattern characters.

Discussion

The most obvious and almost trivial question which comes to mind in the present case is : are the two taxa, *daplidice* and *edusa*, differentiated at the species rank ? There are strong arguments to substantiate the hypothesis of distinction at this level.

The low degree of genetic differentiation among local populations within both taxa is an indication of a relatively high gene-flow between these populations. This is in good accordance with the generally accepted opinion on the biology of *Pontia daplidice* as being a migrant. The population-genetical data suggest that both entities, *daplidice* and *edusa*, share the same behaviour, which is also substantiated by field observations. They may therefore be classified among the species with an "open" population structure.

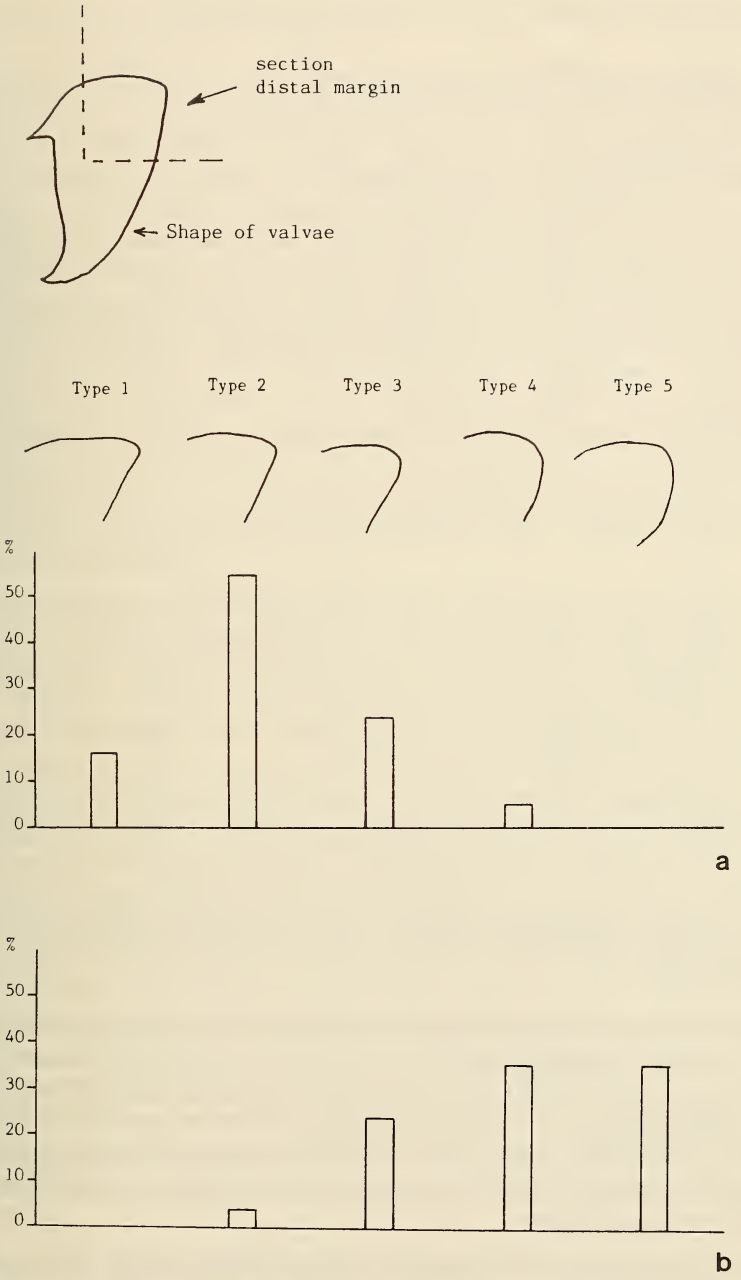


Figure 4. Shape of distal margin of valvae (male genitalia). 4a : *Pontia edusa* (n = 45). 4b : *Pontia daplidice* (n = 75).

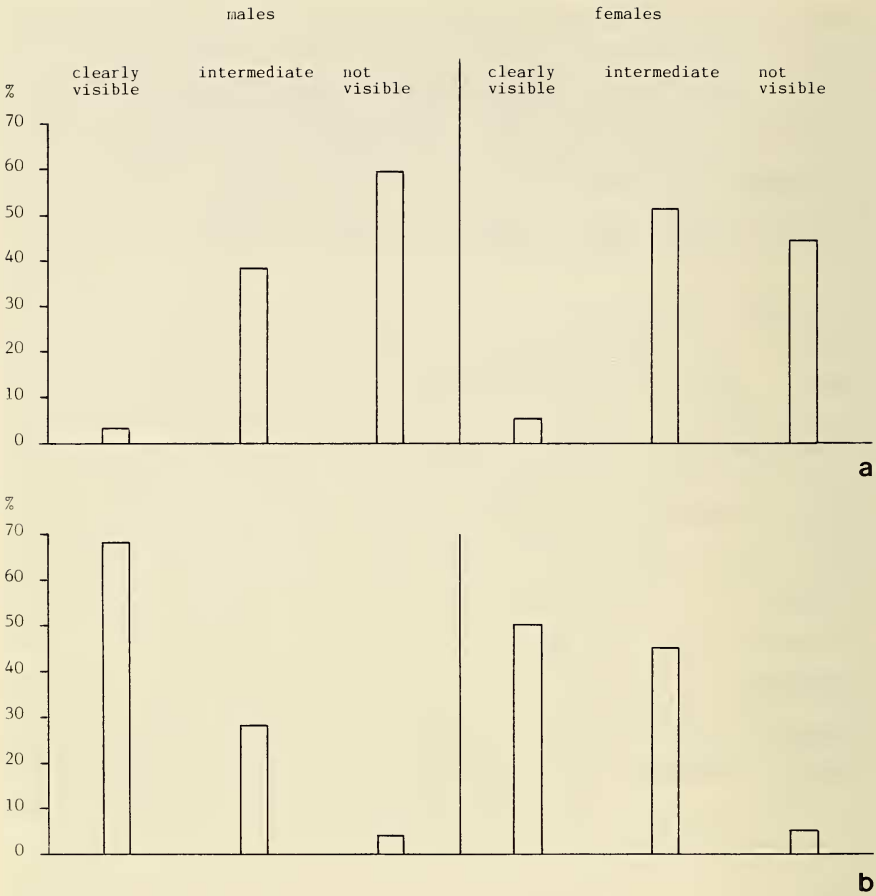


Figure 5. Visibility of submarginal black spot on forewing underside between vein 3 and 4. 5a : *Pontia edusa* : males (n = 45), females (n = 44). 5b : *Pontia daplidice* : males (n = 75), females (n = 54).

Under these conditions, the lack of heterozygous individuals at the diagnostic loci is strongly suggestive that the two taxa are genetically isolated. This observation is reinforced here by means of first intertaxa hybridization experiments that show a strong postmating barrier which acts as early as in the F_1. This barrier seems not to allow any embryonic development in pairings of *daplidice* males with *edusa* females and leads to reduced viability in the reciprocal crosses.

The fact that there is no unequivocal morphological character that allows to identify the two taxa cannot be regarded as an argument against separation at species level between them. Scores of examples where speciation is not

accompanied by morphological differentiation are already known in insects, and the present one is just one more.

A major difficulty in ranking *daplidice* and *edusa* is caused by the fact that at present no data for sympatric populations are available. In such situations it is not possible with any method to prove interruption of gene-flow or, in other words, the existence of two species. Even a large genetic distance as shown here by means of biochemical-genetic methods is only a strong indication for speciation (BERLOCHER, 1984 ; GEIGER, in press). Moreover, the crosses described here have been undertaken with individuals originating from localities too distant to provide conclusive results. In other cases, where obviously a specific continuum is involved, crosses between geographically distant populations often give rise to "hybrid breakdown" phenomena (OLIVER, 1979 ; LORKOVIC, 1986). However, in a large study on the genetic relationships of Pieridae, comprising over 120 taxa, such a large genetic distance as reported here for *daplidice* and *edusa* has never been found for conspecific taxa in any comparisons involving taxa whose systematic rank is currently not questioned (GEIGER and SCHOLL, 1985 ; GEIGER, in press, and unpublished results).

Actually the only undebatable criterion for species rank is that proposed by CUÉNOT (1936) : sympatry with no genetic exchange. The present study indicates that *daplidice* and *edusa* have a good chance of contact in at least two regions : i) between Nice and Genoa, and ii) in south-eastern Turkey (see also WAGENER, 1988). The structure of such a contact zone, if it occurs, is of utmost interest as most taxa described with weak premating, but strong postmating barriers entering contact have been of the "closed" population structure type. The result was very often a parapatric type of distribution – for instance in the group of *Erebia tyndarus* (DE LESSE, 1960). In such a zone the genetic losses associated with the inviability of the hybrids are limited to a narrow region. In the present case, the high potential for dispersion of the individuals of both species should extend the zone where both taxa exert a "hybridisation load" upon each other. This is obviously not a stable situation and we may well be faced with a much more interesting topic than deciding whether *daplidice* and *edusa* belong to the same species. Did they adapt to such an unstable situation by eliminating the incompatibility factors observed, or by developing premating barriers ? Or does the "hybridisation load" persist, resulting in a zone of instability or even a gap in the distribution area of both taxa, or is one taxon eliminating the other one ? Or is something else occurring ?

Any individuals from regions where a contact is to be expected will be highly welcome for electrophoretic analysis and collectors are invited to send freshly collected specimens alive in a paper triangle to one of these author's

addresses. As enzyme electrophoresis is still the only means to unequivocally identify the two potential species, we are also ready to analyse individuals from other places, especially those from localities distant to those reported here.

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